REPORT DOCUMENTATION PAGE

Form Approved
OMB NO. 0704-0188

1. AGENCY USE ONLY (Leave Blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	05/1997-04/2000	FINAL 05/1997-04/2000

4. TITLE AND SUBTITLE
Organophosphorus surface chemistry in organized media

5. FUNDING NUMBERS
DAAG55-97-1-0160

6. AUTHOR(S)
Roger M. Leblanc

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Department of Chemistry, University of Miami
Coral Gables, Florida 33124

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U. S. Army Research Office
P.O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSORING / MONITORING AGENCY REPORT NUMBER
ARO 36479.8-CH

11. SUPPLEMENTARY NOTES
The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision, unless so designated by other documentation.

12 a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited.

12 b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

Principal objective was to understand the molecular interactions between organophosphates and the enzymes that are inhibited by organophosphates or enzymes that catalyze the hydrolysis of organophosphates, at the air/water interface (Langmuir films) and in Langmuir-Blodgett (L-B) films and to develop a biosensor with greater sensitivity. This report contains the data on detailed study of acetylcholinesterase (AChE) Langmuir films and molecular interactions between AChE and OP compounds in monolayers and in Langmuir-Blodgett films. Data demonstrates that acetylcholinesterase forms a highly stable monolayer at the air/aqueous interface. Brewster angle microscopy data indicate the reversible formation of domains upon compression and decompression of the enzyme monolayer at the air/aqueous interface. The tapping mode atomic force microscopy (TMAFM) data on L-B films of AChE indicate abundant globular AChE monomers and a limited number of large and medium sized tetramer forms of AChE. The TMAFM images and UV-vis and FTIR spectroscopic data suggest that the configuration of the enzyme was completely modified in the presence of the paraoxon and the ellipsoidal shape of AChE disappeared. As a continuation of this project, work was initiated on designing and testing of a biosensor using fluorescence labeled AChE monolayers and on characterization of Langmuir andLangmuir-Blodgett films of organophosphorus acid hydrolase and its interactions with organophosphorus compounds.

14. SUBJECT TERMS
Organophosphorus compounds, acetylcholinesterase, organized monolayers, biosensor, organophosphorus acid hydrolase, Langmuir films, Langmuir-Blodgett films

15. NUMBER OF PAGES
19

16. PRICE CODE

17. SECURITY CLASSIFICATION OR REPORT	18. SECURITY CLASSIFICATION ON THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UL

FINAL PROGRESS REPORT

(May 1997-April 2000)

Roger M. Leblanc
Department of Chemistry
University of Miami
Coral Gables, FL 33124

Foreword

Organophosphorus (OP) compounds enjoy a broad commercial market both as insecticides and therapeutic agents. Besides these they are also used as chemical warfare agents as the compounds such as sarin and soman are toxic nerve agents and are deadly to the living beings. With the ratification of Chemical Convention Treaty, it became obligatory to destroy the existing stock piles of these deadly gases. Now, more than ever there is a need for the highly sensitive detection and efficient decontamination system for these compounds in traces in order to keep our environment free of these deadly toxins. In an attempt to improve the sensitivity of the detection systems, Army Research Office has funded our research proposal on molecular interactions in mono-molecular films between OP compounds and the enzymes that are inhibited by or that catalyze the oxidation of OP compounds. As an initial attempt we investigated the interactions between acetylcholinesterase (AChE) and an OP compound (paraoxon) in monolayers. The advantage of using monolayer technique is the possibility of developing organized monomolecular layers, in order to have the catalytic sites of the enzymes exposed to substrates or inhibitors. Such an organized monolayers of enzymes, if used in detection systems could enhance its sensitivity. Our investigations on AChE during the past three years were presented in this report. We are continuing this project work as our research funding was renewed for three more years. Research work on designing and developing a sensitive detection system is very fascinating and our group is excited to continue this work.

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Statement of the Problem Studied

Organophosphorous (OP) compounds are known to cause acute toxic effects in animals and human beings. Their mode of action has been ascribed to their ability to inhibit acetylcholinesterase. Hence, sensitive, rapid and reliable detection and decontaminant systems are very important for the protection of environment and human health. Biosensors for the detection of the OP compounds, using acetylcholinesterase (AChE) activity have been developed. These sensors used a variety of transducers. None of these sensors have used organized molecular layers of AChE. We believe that certain characteristics such as the response time, sensitivity and reusability of the sensor can be improved by using Langmuir-Blodgett film technology. Further, in order to develop a suitable decontaminant system, a thorough understanding of the nature of molecular interaction between OP compounds and their target enzymes or the enzymes that catalyze the hydrolysis of these OP compounds is necessary. Hence, the ***principal objective*** was to understand the molecular interactions between OP compounds and the enzymes that are inhibited by them or enzymes that catalyze hydrolysis of these compounds, at air/water interface (Langmuir films) and in Langmuir-Blodgett (L-B) films and to develop a biosensor with greater sensitivity.

Summary of the Most Important Results

I. Characterization of Langmuir films of acetylcholinesterase:

The surface pressure-area (π-A) isotherm of AChE monolayer compressed on the surface of a salt containing subphase is presented in Figure 1. A collapse surface pressure appears at about 35 mN/m and an apparent limiting molecular area of 11,000 $Å^2$ is observed. An inflection point on the AChE isotherm was observed at a surface pressure around 16 mN/m. We also recorded the π-A isotherms obtained when AChE monolayer compressed on three different subphases containing different electrolytes (either NaCl, KCl, or $CaCl_2$). There was no significant difference when the electrolyte of the bulk subphase was substituted. Further, the effect of pH on the AChE monolayer was also investigated. Absorption spectra of the enzyme monolayer recorded at different surface pressures directly at the air/water interface (10 mM KCl, 0.1 M KH_2PO_4 and 0.1 M NaOH, pH 6.5) show an increase in the intensity of the absorption peak at 200 nm. This suggests the formation of a stable monolayer.

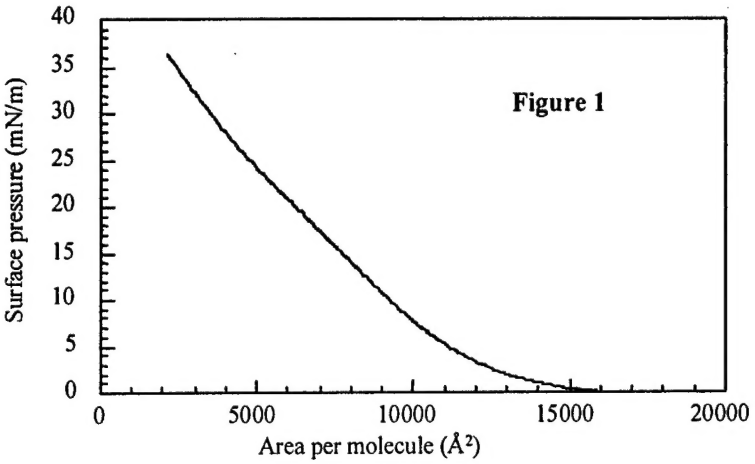
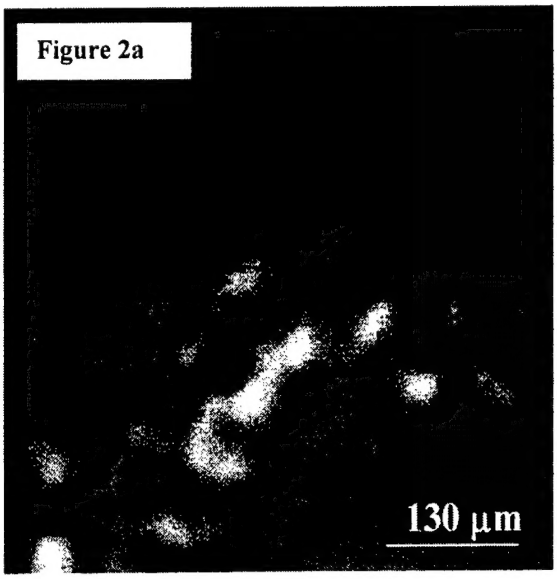


Figure 1

Our data demonstrate that acetylcholinesterase forms a highly stable monolayer at the air/aqueous interface. This remarkable stability of the enzyme may be attributed to its polar and non-polar nature, and also to its high molecular weight. Studies on the pH dependent process of the enzyme stability showed that the organized AChE monolayers at the interface require a pH of 6.5 in the bulk medium. The results obtained at pH values below the isoelectric point can be attributed to the aggregation of the enzyme and loss of the molecules in the subphase. The absorption spectrum of the enzyme monolayer can be considered as an evidence of the organization of AChE molecules at the air/aqueous interface.

We examined further the topography of Langmuir and Langmuir-Blodgett films of AChE using Brewster angle microscopy and atomic force microscopy.

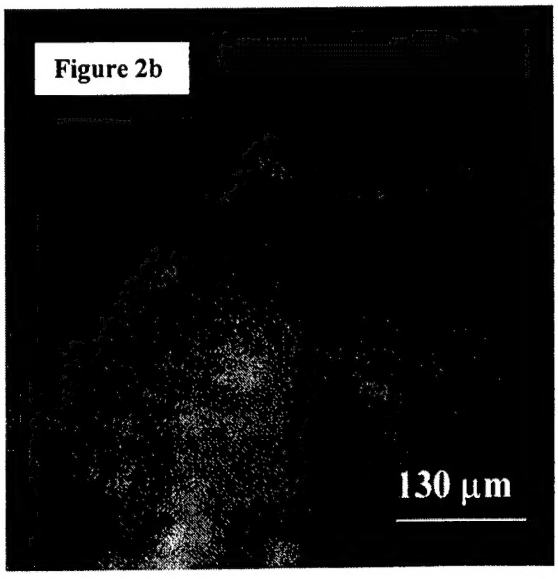
II. Surface topography of Langmuir and Langmuir-Blodgett films of AChE:



a) Brewster angle microscopic (BAM) study of Langmuir films of AChE:

The images recorded using the BAM were analyzed at two different surface pressures (0 mN/m, Figure 2**a** and 20 mN/m, Figure 2**b**) and during the decompression of the monolayer. In all images, we noted areas with different brightness due to variations in molecular density, which correlates with the thickness of the monolayer at the interface. During the compression of the monolayer (from a molecular area of $1.5x10^2$ Å^2 to $1x10^4$ Å^2) and at zero surface pressure (Figure. 2**a**), we observed different domains with different sizes dispersed at random on the aqueous surface. These Brewster angle microscopic data agree with the surface potential results in the fact that at large molecular area, i.e. no surface pressure, there is formation of islands on the aqueous subphase.

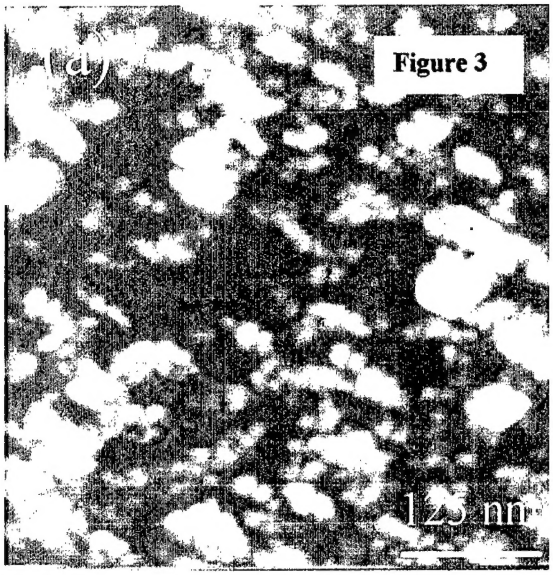
Upon compression, the domains start growing and the patches become larger (Figure 2**b**). The dark areas disappeared and large domains covered almost all the surface. Therefore, a surface pressure of 20 mN/m, which corresponds to the plateau value of the surface potential, can be considered as the end point of the liquid expanded phase and the beginning of formation of a more condensed enzyme monolayer.

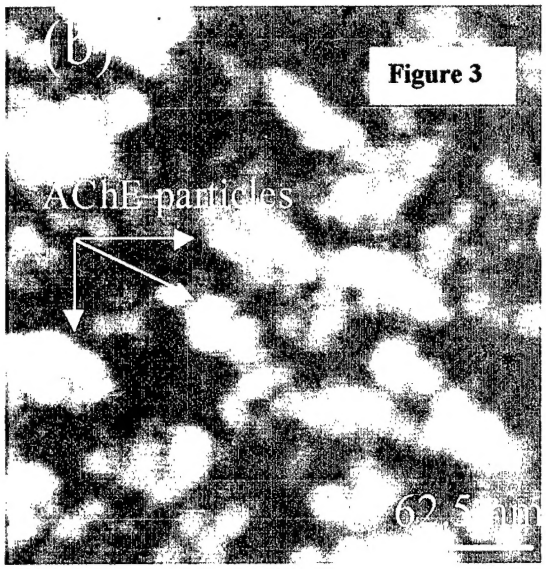


Brewster angle microscopic images recorded during the decompression of the monolayer showed the break down of the domains on the surface. The BAM data indicate the reversible formation of domains upon compression and decompression of the enzyme monolayer at the air/aqueous interface.

b) Atomic force microscopic study of L-B films of AChE:

AFM images revealed (3**a, b)** that AChE particles can be grouped into 3 different categories. The larger and medium sized particles are shaped like an ellipsoid and their dimensions with respect to the ellipsoid axis are 68-84 x 32-44 x 7-8 nm and 36-48 x 21-26 x 2-3 nm, respectively. The small particles of AChE are the most abundant population of the L-B film and their average size is 25 x 18 x 1.6 nm.

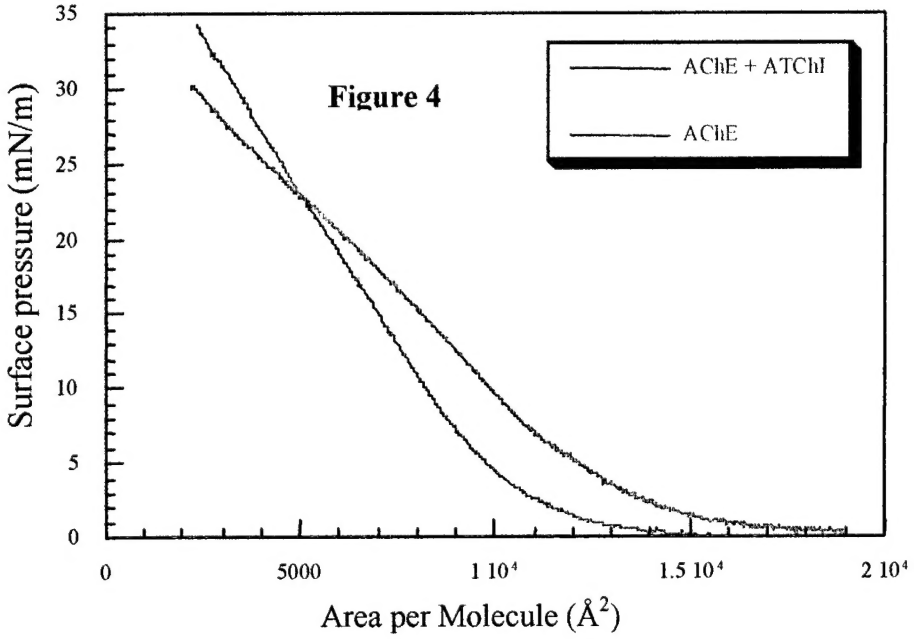




The ellipsoidal shape of AChE particles observed with the TMAFM is in agreement with the three dimensional structure determined by X-ray analysis. As mentioned, globular AChE molecules, in solution, might exist in the form of monomer, dimer or tetramer. Considering this observation, the size of the small particles are within the range of the dimensions estimated by X-ray data for an AChE monomer (175,000 $Å^3$). On the other hand, the dimensions obtained with the AFM (720,000 $Å^3$) for the large and medium size of AChE particles are within the range of the estimated dimensions of an AChE tetramer (700,000 $Å^3$), using the monomer dimensions obtained by the X ray analysis. *Therefore, we consider that the most abundant population of the L-B film is the globular AChE monomer. Moreover, the larger and medium sized particle of AChE represent the tetramer form of this enzyme.*

III. Molecular interactions between acetylcholinesterase and acetylthiocholine (substrate) or organophosphate at the air/aqueous interface:

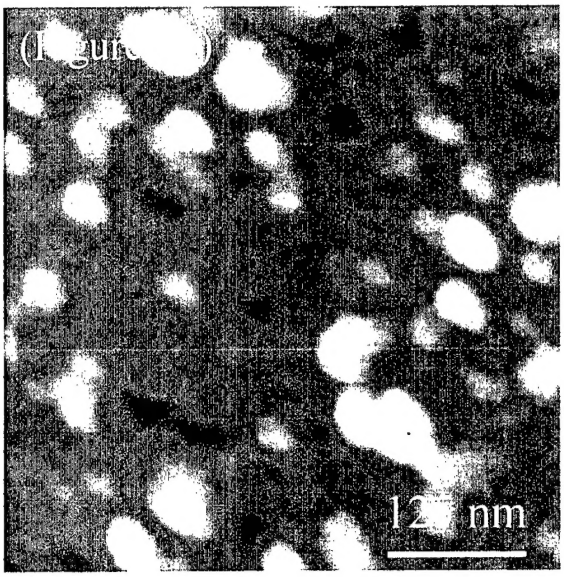
We made a comparative study of AChE interaction with substrate, acetylthiocholine (ATChI) and the OP compound, paraoxon. A comparative study between the surface pressure-area isotherms of AChE compressed in the presence and in the absence of the substrate in the subphase (Figure 4) shows a significant difference in the apparent limiting molecular area. The collapse surface pressures of AChE monolayers are 30 and 35 mN/m in the presence and the absence of the ATChI in the subphase, respectively. An increase of 3,000 $Å^2$ in the limiting

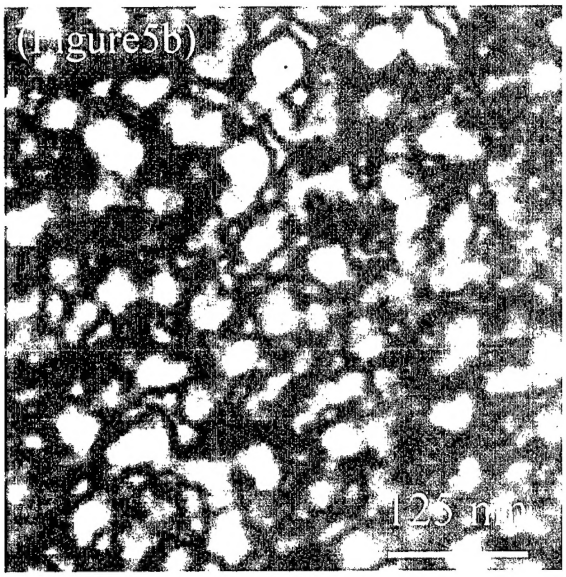


molecular area is observed when the ATChI is added to the subphase and is due to the interaction between the substrate and the enzyme at the air/aqueous interface.

Similarly the surface pressure-area isotherm of the AChE monolayer in the presence of the paraoxon in the subphase is significantly different from that of the one in its absence. The limiting molecular areas correspond to 11,000 and 4,500 $Å^2$ in the absence and the presence of the paraoxon, respectively. The collapse surface pressure is not higher than 16 mN/m in presence of the organophosphate.

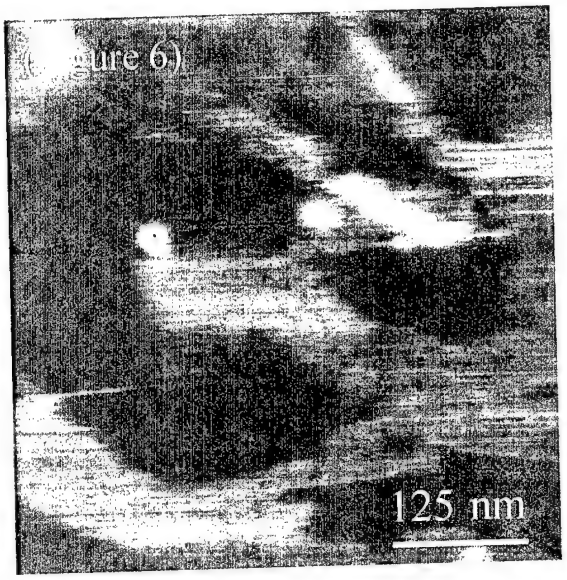
The topography of the Langmuir-Blodgett films was recorded by tapping mode atomic force microscopy (TMAFM). For a better understanding of the complex formation mechanism





between AChE and ATChI, the AChE monolayer was prepared and examined with TMAFM in two steps. The monolayer was first compressed on the substrate-free buffered subphase. Once a surface pressure of 25 mN/m was reached, the acetylthiocholine was injected into the subphase. The TMAFM images (Figure 5**a**) of a transferred monolayer, six minutes after the injection, show the presence of acetylcholinesterase-acetylthiocholine complex and a homogeneous monolayer composition. However, the images of a transferred monolayer at the same surface pressure, but 15 minutes after the injection, showed the formation of a mixed monolayer due to the presence of both the enzyme-substrate complex and free enzyme (Figure 5**b**). Compression of the AChE monolayer on a substrate subphase indicated occurrence of the hydrolysis reaction at the interface and ended before a surface pressure of 25 mN/m was reached. ***Therefore, the topography of a monolayer prepared on a subphase containing the substrate resulted in a heterogeneous surface structure due to the presence of free enzymes and reaction products. UV-Vis data confirmed the observations deduced from the TMAFM images.***

The structural conformation of the enzyme was altered significantly by the presence of the paraoxon. Large domains were observed rather than an organized acetylcholinesterase monolayer, and the spectroscopic properties indicated that the interaction between the acetylcholinesterase and the paraoxon took place at the air/aqueous interface (Figure 6). As it is seen in Figure 6, the structure of the monolayer is completely different from the one obtained in the absence of the inhibitor (Figure 5a). No sign of the particle distribution was noticed.



As it is evident from our results on Langmuir films of AChE in the presence and in the absence of paraoxon in the subphase, there is a decrease of 6,500 $Å^2$ in the limiting molecular area. The change in the surface pressure-area isotherm of the AChE monolayer suggests the binding of the paraoxon to the enzyme molecule causing a degradation and dissolution of the enzyme into the subphase. The TMAFM images indicate that the configuration of the enzyme was completely modified in the presence of the paraoxon and the ellipsoidal shape of AChE disappeared.

IV. Polarization Modulated FT-IR Spectroscopy Studies of Acetylcholinesterase Secondary Structure at the Air-Water Interface:

Polarization modulation infrared reflection absorption spectroscopy (PMIRRAS) was used to measure the vibrational spectrum of acetylcholinesterase (AChE) at the air-water interface in its free form and bound to either its substrate, acetylthiocholine, or organophosphorous and carbamate inhibitors.

a. Secondary structure of AChE at the air-water interface:

The PMIRRAS spectra of the AChE monolayer showed amide I and II bands which are consistent with the presence of both α helical and β sheet conformations. Based on both experimental and theoretical calculations, the 1655 cm^{-1} frequency was assigned to the α helical conformation while the one at 1630 cm^{-1} is associated with the anti-parallel chain pleated sheet conformation (β structure). X-ray data show that α helices of the AChE can be grouped into two populations; short α helices with 14 residues or less and longer α helices containing 15 residues or more. In the present work, the two populations of α helix were distinguished at higher surface pressures using the PMIRRAS technique. At 35 mN/m, the enzymes were well packed and the

secondary structure components were being oriented more vertically so that all the frequencies could be detected at the interface using the PMIRRAS technique.

The analysis of the amide I and II bands showed no major spectral changes of the enzyme conformation at the interface. Application of higher and lower surface pressures did not affect significantly the shape and the position of the amide bands. The qualitative analysis showed that secondary structure of AChE monolayer consists mainly of both the α helical and the β sheet conformations. Furthermore, two classes of α helices (short and longer helix) were distinguished at the air-water interface using the PMIRRAS technique. This phenomenon was predicted theoretically earlier and was observed experimentally in solution.

b. AChE-substrate interaction at the air-water interface:

The enzyme-substrate interaction was studied at the air-water interface by spreading the enzyme as monolayer and dissolving the substrate, ATChI, into the subphase. The hydrolysis reaction was investigated when the enzyme solution was spread at nil surface pressure. PMIRRAS spectra were collected at different surface pressures and the signal increased during the compression. At zero surface pressure, only α helix component was seen at 1655 cm^{-1}. At 5 mN/m, the α helix shifted to higher frequency (1659 cm^{-1}). Changes in the enzyme secondary structure occurred after compression of the AChE monolayer to 5 mN/m in the presence of ATChI in the subphase. These conformational changes are reversible and are only necessary to position the substrate for subsequent intramolecular interactions. Compression to higher surface pressure (30 mN/m) led to the apparition of both the α helix and the β sheet structures at 1655 and 1630 cm^{-1}, respectively. After compressing the enzyme to 5 mN/m, overlapping bands were observed around 1160-950 cm^{-1} and are associated with the presence of the substrate occupying the enzyme active site at the interface. At higher surface pressures, the intensity of the bands increased due to the increase in the surface concentration of the enzyme and the substrate at the interface. The bands at 1074 and 1128 cm^{-1} are associated with the presence of the reaction product, thiocholine iodide close to the interface.

PMIRRAS data indicated the presence of both the substrate and the reaction products at the interface ten minutes after spreading of the enzyme at high surface pressures. In fact, at 5 mN/m, the substrate binding and formation of a complex AChE-ACTh is illustrated by C-O-C vibration mode. This is in agreement with the tapping mode atomic force microscopy (TMAFM) images and the UV-Vis data. TMAFM images of a transferred film 10 minutes after the substrate injection beneath the AChE monolayer indicated the presence of an AChE-ACTh complex.

c. AChE-inhibitors interaction:

Molecular interaction of the AChE with the paraoxon was studied at the air-water interface by dissolving the paraoxon in the subphase at a concentration of 4×10^{-4} M and spreading the enzyme as a monolayer. At first glance, the AChE secondary structure was drastically destroyed

upon the paraoxon binding to the enzyme (Figure 7). The obtained spectra did not have the shape and the position of the amide I and II bands. The amide I band position shifted to higher frequency components and the structure of the amide II band was significantly altered by the presence of the inhibitor and vanished at the collapse surface pressure. The data indicate that the enzyme was completely unfolded when it was compressed on a subphase containing the paraoxon. This agreed with the TMAFM results, where an unfolding of the AChE was observed when the enzyme was compressed on a subphase containing the paraoxon.

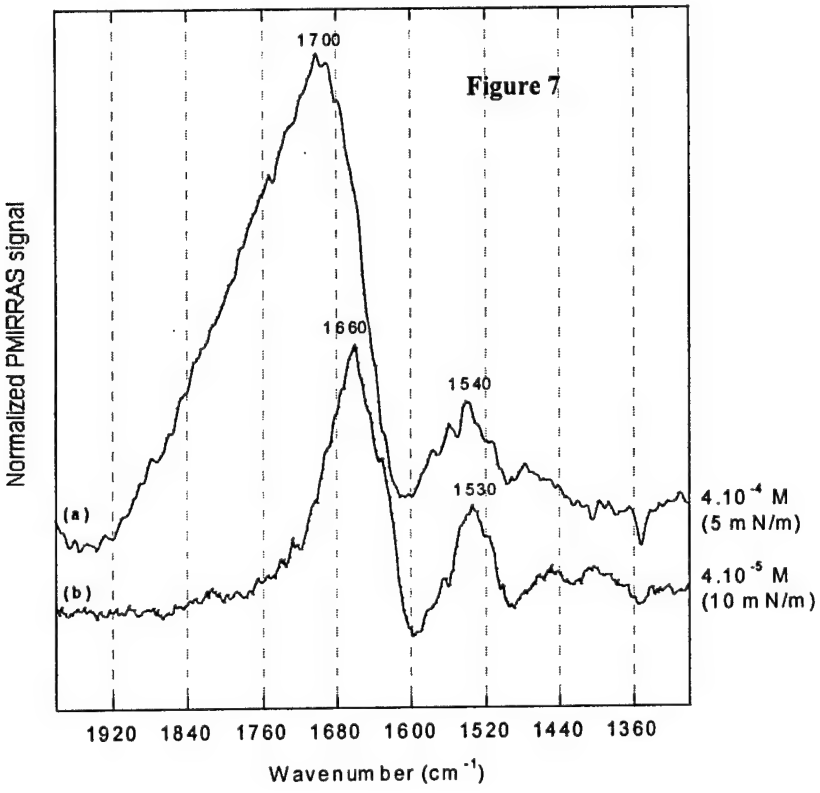


Figure 7

The inhibitory effect of an another organophosphate, phenylmethylsulfonylfluoride (PMSF), on the AChE was not as significant as by the paraoxon where the secondary structure was completely destroyed.

d. Reactivation of the paraoxon inhibited AChE:

The paraoxon inhibited-AChE was reactivated by injecting the nucleophilic agent [trimethyl bis –(4 formylpyridinium bromide)dioxime]TMB-4 beneath the inhibited-AChE monolayer. From the PMIRRAS data, we noticed that the AChE secondary structure might be re-established thirty minutes after the dioxime injection following the inhibition by the paraoxon. For the first time, we observed these changes in the enzyme secondary structure after a reactivation following the paraoxon enzyme inhibition. In fact, the reactivation mechanism occurred via a phosphorylation of the dioxime resulting, therefore, in a dephosphorylation of the enzymes which were liberated free into the subphase.

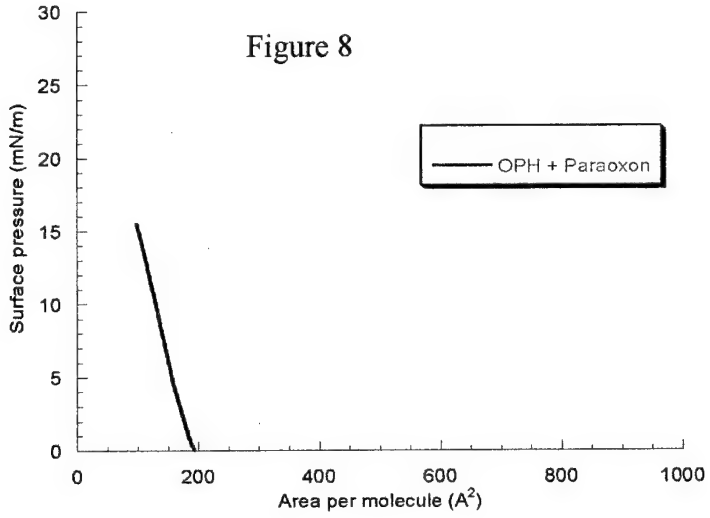
We also characterized the Langmuir films of organophosphorus hydrolase, an enzyme that catalyzes the hydrolysis of OP compounds. Organophosphorus hydrolase was provided by Dr. T. C. Cheng and Dr. Joseph DeFrank at U.S. Army Edgewood Research, Development and Engineering Center, Maryland 21010.

V. Characterization of Langmuir films of organophosphorus hydrolase (OPH):

Our experiments demonstrate that the OPH monolayer is highly stable at the air-water interface as observed with AChE. Compression and decompression cycles have shown that the OPH monolayer can be compressed and decompressed several times without any collapse or change in the limiting molecular area. On each compression/decompression cycle the increasing and decreasing rates are equal. Moreover, when the surface pressure was held to 15 mN/m, the area remained constant without showing any significant change for 120 minutes. No desorption of the enzyme from the air-water interface has occurred. The surface potential corresponds to the measurement of the orientation of molecular dipole moment at the air-water interface. The fluctuations recorded at large molecular area indicate the presence of different domains at the interface. At higher compression, these fluctuations became low and an increase of the surface potential was observed. This is due to the fact that as the surface was compressed, these domains became closer and closer, and finally formed the monolayer. The surface potential reached its maximum and remained constant until the monolayer collapsed. Moreover, the increase of the surface pressure when the surface potential was constant to its maximum provides evidence that a stable and closed-packed enzyme monolayer has been formed. The BAM data agree with the surface potential results and indicate the reversible formation of domains upon compression and decompression of the enzyme monolayer at the air/aqueous interface as was observed with AChE.

The interactions between OPH and paraoxon were studied by spreading the enzyme at the surface of the paraoxon dissolved buffered subphase. The two isotherms, presented in the figure 8, are significantly different. The results suggest that there are interactions between OPH

Figure 8



Surface pressure-area isotherms of OPH monolayers at the air-water interface in the absence and in the presence of paraoxon in the subphase.

and paraoxon occurring at the air-water interface and cause degradation and/or dissolution of the enzyme in the subphase.

VI. Design and characterization of a Biosensor:

As the L-B film technology allows the creation and deposition of oriented protein films with high density of active sites of the enzyme molecules and a reproducible casting of the receptor layer, we are trying to apply this L-B film methodology to characterize a prototype sensor using L-B films of AChE. As part of our objectives of our proposal, we worked on developing a prototype of a fiber optic biosensor using L-B film technology. The measuring principle involved in this study is very simple. Paraoxon binds to AChE and inhibits its activity. Upon binding of paraoxon to AChE, changes in the fluorescence properties of attached fluorescent probe FITC (Fluorescein isothiocyanate), will be monitored.

Currently, we are trying to transfer the monolayers of AChE onto the core surface of the fiber. The chemical treatment of the core and the Langmuir-Blodgett technique allow achieving a homogeneous film with a single molecular thickness. Since the latter displays a key-role in targeting the analyte, they are consequently likely to be much more available for a selective recognition.

The first stage of the experiments consisted of dipping the coated fiber tip in an aqueous solution and recording fluorescence. The band at 525 nm indicates the fluorescence of the biosensing device. The intensity of this band remains constant over a long period of time, which suggests the stability of the coating. During the second stage of the experiments, we added paraoxon in the aqueous solution and repeated the experiments. The fluorescence band at 525 nm totally disappeared. This can be explained by the fact that paraoxon acts as a fluorescence inhibitor. It binds to specific sites of the AChE and prevents the FITC from fluorescing.

We are trying to transfer the organized monolayers of labeled AChE onto the treated optical fiber and record the fluorescence signal in the presence of a substrate or an inhibitor such as paraoxon. We are also trying to entrap the labeled AChE in a gel and study its properties.

VII. Future work:

Work is in progress on characterization of Langmuir and Langmuir-Blodgett films of OPH and organophosphorus acid anhydrase, and on the designing and testing of a biosensor using fluorescence labeled AChE monolayers.

List of all publications and technical reports:

1. Dziri, L., B. Desbat, and R. M. Leblanc (1999). "Polarization-Modulated FT-IR spectroscopy studies of acetylcholinesterase secondary structure at the air-water interface". J. Am. Chem. Soc. 121, 9618-9625.

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List of all participating scientific personnel showing any advanced degrees earned by them while employed on the project

Dr. Roger M. Leblanc	Principal Investigator	One month summer salary
Dr. Germain Puccetti	Postdoctoral Fellow	Worked on 2D sol-gel process for acetylcholinesterase encapsulation (AChE)
Sebastien Vidon	Postdoctoral Fellow	Worked on Inelastic electron tunneling spectroscopy (IETS)
Leila Dziri	Graduate student Received Ph. D.	Worked on surface chemistry and spectroscopy of AChE
Jean-Luc Brousseau	Graduate student Received Ph. D. degree	Worked on IETS study of AChE
Aurelien Pisseloup	Postdoctoral Fellow	Worked on IETS study of AChE
Shaopeng Wang	Graduate student Received Ph. D.	Worked on 2D sol-gel process for biosensor fabrication
David Gonzalez	Postdoctoral Fellow	Worked on enzyme entrapment
Nandini Katipamula	Graduate student Received Masters degree	Worked on 2D sol-gel process for biosensor fabrication
Micic Miodrag	Graduate Student	Worked on ESEM examination of monolayers of OPH

Report of Inventions (By Title only)

Nil

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Appendixes

Nil